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FOREWORD

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5)Introduction

The overall objective of the work proposed in this grant is to provide a detailed understanding of the molecular mechanism by which G2/M regulation is achieved in human cells. In particular we are focusing on how initiation of M-phase is delayed in cells that have been treated with agents that induce DNA damage or that prevent synthesis of DNA. By providing a more detailed explanation of how cytotoxic therapy brings about cell death we hope to provide clinicians with better tools for the treatment of breast cancer. Previous work in human cells showed that the mitosis inducing kinase Cdc2/Cyclin B is inhibited by phosphorylation of threonine-14 and tyrosine-15 [1, 2]. Disruption of these phosphorylation sites abrogates checkpoint-mediated regulation of Cdc2 and renders cells highly sensitive to agents that damage DNA [3, 4]. Phosphorylation of these sites is controlled by the opposing activities of the Wee1/Myt1 [5-7] kinases and the Cdc25 phosphatase [8]. The regulation of these enzymes is therefore likely to be crucial for the operation of the G2/M DNA damage checkpoint. However, evidence that the activity of these enzymes is substantially altered in response to DNA damage is lacking [9]. We have evidence that the activity of the mitotic inducer Cdc25 is decreased following irradiation. We have identified two human kinases that phosphorylate and inactivate Cdc25 *in vitro*. One is the previously characterized Chk1 kinase [10]. The second is a novel human gene with homology to the Cds1/Rad53 [11, 12] family of checkpoint kinases. The results of this study suggest that in human cells, the DNA damage checkpoint involves direct inactivation of Cdc25 catalyzed by Cds1 and/or Chk1.

6) Methodology and Results

As described in a previous annual report (94-95) our analysis of the pattern of expression and activity of mitotic checkpoint control proteins (CDC2, Cyclin A and B and WEE1) in transformed and non-transformed breast cell lines showed that there is over-expression of CDC2/Cyclin B in transformed breast cell-lines (Equivalent to Task 1 in SOW). However the level of over-expression was not very great and we concluded that it would not be feasible to analyse the viability of cells following cytotoxic treatment relative to these modest changes in expression level. We therefore decided to concentrate our efforts in pursuit of technical objectives 2 and 3: That is, to determine mechanism by which human cells prevent progression into M-phase following cytotoxic treatments. We have focused on the DNA damage response elicited by ionizing radiation and we have used HeLa cells as a model of p53 minus cancer [13].

In last years report we described the work we had done to look at changes in Cdc25 activity in response to irradiation. We have continued this work and improved the evidence that Cdc25 is down-regulated in response to DNA damage. We have shown that the irradiation induced decrease in Cdc25 activity is suppressed by wortmannin, an inhibitor of phosphatidylinositol (PI)-3 like kinases, and is dependent on the function of the Ataxia Telangiectasia gene. Moreover, we have identified two human kinases that phosphorylate and inactivate directly Cdc25 *in vitro*. One is the previously described Chk1 kinase. The second is a novel human gene with homology to the Cds1/Rad53 family of checkpoint kinases.

Dephosphorylation of Cdc2 is inhibited by DNA damage

The possibility that dephosphorylation of Cdc2 is down-regulated in the presence of DNA damage was investigated. Three distinct Cdc25 proteins are expressed in human cells [14-16]. Microinjection of specific antibodies coupled with distinct patterns of activation suggest that Cdc25A primarily has a role in the G1/S transition [17, 18], and that Cdc25B and Cdc25C primarily have roles in the G2/M transition [19-21]. The exact contribution of Cdc25B and Cdc25C to M-phase progression is not known, therefore, an assay that allows direct analysis of the net phosphatases acting on Cdc2/Cyclin B was used to determine how Cdc2 is maintained in its phosphorylated state following irradiation. In the presence of EDTA the activity of Cdc2/Cyclin B from asynchronous HeLa cell extracts was found to activate over time (Fig. 1a). Activation correlated with loss of the inhibited-phosphorylated form of Cdc2, visualized as the slower migrating species on SDS-PAGE, (Fig. 1b). There was no increase in the levels of Cdc2 or Cyclin B protein, phosphorylation by Wee1 and Myt1 was blocked by the presence of 10mM EDTA. Activation was prevented by vanadate, an inhibitor of Cdc25 and other tyrosine phosphatase. Furthermore, immune-depletion with Cdc25C specific anti-sera showed that activation of Cdc2/Cyclin B was dependent on the presence of Cdc25, (Fig. 1a). Thus, activation of Cdc2 was the result of its dephosphorylation and is a measure of total Cdc25 activity in the extract. In lysates of asynchronous HeLa cells the endogenous Cdc25 phosphatase activity is sufficient to dephosphorylate and activate more than 80% of the available Cyclin B/Cdc2 in 30 minutes (Fig. 1a and b). Analysis of lysates of HeLa cells in which the DNA had been damaged by exposure to 10 Gray of γ -irradiation one hour before harvesting showed a significant reduction in the rate of activation of Cdc2, such that less than 25% of the available Cdc2/Cyclin B was activated during the 30 minutes incubation. The amount of Cdc2/Cyclin B in complex was not significantly altered and it was activated to the same extent as control

Cdc2/Cyclin B by addition of exogenous GST-Cdc25C(Fig 1a). Irradiation with 10 Gray led to more than 3-fold reduction in the rate of Cdc2 dephosphorylation in the 10 time courses examined. Similar experiments were used to determine if Cdc25 is down-regulated in response to agents that block DNA synthesis. We found no evidence that Cdc25 activity is down-regulated in response to a number of agents that block DNA synthesis (data not shown).

Radiation induced inactivation of Cdc25 requires the activity of PI 3-like kinases

If the inactivation of Cdc25 measured above is part of the DNA-damage checkpoint response in human cells, then experimental conditions that over-ride the DNA damage checkpoint might be expected to block radiation induced-inhibition of Cdc25. Genetic data from a number of organisms has identified a family of related kinases that are required for DNA damage responses [22]. Structurally these enzymes are related to the PI-3 kinases and at least one member of the family, DNA-Protein Kinase, has been shown to be sensitive to wortmannin [23] *in vitro*. The possibility that a wortmannin-sensitive kinase is upstream of the radiation induced delay in M-phase entry was therefore tested [24]. HeLa cells can be arrested in M-phase by nocodazole, irradiation causes cells to delay in G2 prior to the nocodazole-sensitive M-phase block point. Thus, by scoring the mitotic index of cells that are cultured in nocodazole, it is possible to determine whether entry into mitosis has been delayed. Control cells cultured in the presence of nocodazole for 14 hours, contained 60% mitotic cells, the presence of wortmannin had little effect on this number (Fig. 2a). However, irradiation reduced the number of cells that reach the nocodazole block point to 10%. By contrast, irradiation in the presence of wortmannin had only a modest effect on the number of cells that reach the nocodazole block point (Fig. 2a). These results demonstrate that wortmannin over-rides the DNA damage G2 checkpoint in HeLa cells. The effects of wortmannin on the radiation induced inactivation of Cdc25 was therefore tested. Wortmannin had a minor effect on the activation of Cdc2/Cyclin B in extracts prepared from unirradiated cultures, however, wortmannin greatly diminished the irradiation-induced decrease in Cdc25 activity, (Fig. 2b).

A second experimental condition in which the radiation-induced G2 checkpoint is overridden was provided by cell-lines derived patients with the genetic disorder ataxia telangiectasia. Ataxia Telangiectasia mutant (ATM) cells are defective in both the G1 and G2 checkpoints following exposure to many, but not all, agents that damage DNA [25-27]. The failure of AT-deficient cells to delay in G1 correlates with a failure to up-regulate p53 [28] and with a failure to phosphorylate and activate cAbl [29, 30]. The molecular basis of

the failure to delay in G2 is not known. AT-deficient cells show greatly reduced responses to agents that generate chromosomal breaks such as ionizing γ -rays. Remarkably, AT-deficient cells have near normal responses following the base damage that is generated by irradiation with a UV source [27, 31, 32]. Therefore the effects of UV and γ -irradiation on the Cdc25 activity of AT-plus and AT-minus SV40 transformed human fibroblast cell-lines was investigated (Fig. 2c). AT-minus cells respond to UV-irradiation with a robust reduction in the rate at which Cdc2 is dephosphorylated. By contrast, γ -irradiation had only a modest effect on the rate of dephosphorylation of Cdc2. In AT-plus cells the rate of dephosphorylation of Cdc2 was significantly reduced following either ionizing-radiation or UV-radiation. Samples in which Cdc2/Cyclin B was activated by addition of exogenous GST-Cdc25 showed that both cell types contained similar quantities of Cdc2/cyclin B following irradiation. These data show that the ATM gene product is required for the efficient inactivation of Cdc25 following γ -irradiation and demonstrate a correlation between inactivation of Cdc25 and delayed entry into M-phase following DNA damage [25-27].

Identification of a human homologue of fission yeast checkpoint kinase Cds1

An important objective of this work was to identify potential mediators of the checkpoint response in human cells. Potential mediators of the checkpoint dependent inactivation of Cdc25 were searched for by identifying human homologues of known yeast checkpoint genes. In view of the similarity of mitotic control mechanisms in fission yeast and mammals our attention focused to two kinases, Chk1 and Cds1, that are required for the DNA damage and replication checkpoint in fission yeast [12, 33]. In collaboration with Dr. Andy Parker of Janssen Research foundation, (Beerse, Belgium) an expressed sequence tag (EST) with significant homology to the *cds1* gene of *S. pombe* was identified. The human cDNA predicts a translation product of 543 amino acids with a molecular weight of 61 kDa. The predicted HsCds1 protein is 28% identical to the *cds1* protein of *S. pombe*, 28% identical to RAD53 and 27% identical to the DUN1 kinase of *S. cerevisiae*. Sequence alignment of these homologs (Fig. 3a) shows several regions of homology outside the kinase domain, including conservation of the Fork Head Associated domain [34]. The human protein shows the same overall structure as *cds1* and Dun1 in that it lacks the long C-terminal extension found in Rad53. Northern blot analysis with HsCds1 identified a single transcript of ~2.2 kb expressed in testis and in 8 human cancer samples examined (Fig. 3b).

6his-Cds1 and 6his-Chk1 phosphorylate and inhibit Cdc25 *in vitro*.

To facilitate biochemical analysis 6his-Cds1 and 6his-Chk1 were expressed in insect cells, affinity purified and incubated in extracts of HeLa cells in the presence of an ATP-regenerating system. After 30 minutes at 30°C EDTA was added, to inhibit kinases in the extract, and the rate of dephosphorylation and activation of Cdc2/Cyclin B was monitored as in Figure 1. Both 6his-Cds1 and 6his-Chk1 were found to significantly reduce the activation of Cdc2/Cyclin B in these assays (Fig. 4a). The reduced activation of Cdc2 was dose dependent and required ATP (not shown). Confirmation that Cdc2 was not irreversibly inhibited by 6his-Chk1 or 6his-Cds1 was shown by the activation that resulted when excess GST-Cdc25C was added after kinase treatment. Thus, both 6his-Cds1 and 6his-Chk1 can mimic the radiation induced down-regulation of Cdc25 seen in extracts. These experiments used HeLa cell lysates that had been clarified by centrifugation therefore it seemed unlikely that changes in sub-cellular locale could account for the inactivation of Cdc25 [35]. However, indirect mechanisms of inhibition could not be excluded by this assay. We therefore used affinity purified reagents (Fig 4c) to determine whether 6his-Cds1 or 6his-Chk1 can directly phosphorylate and inhibit GST-Cdc25 activity. GST-Cdc25 was incubated with either 6his-Cds1, mock beads or 6his-Chk1 in the presence of [γ -³²P] ATP for 15 min. at 30°C. Proteins were resolved by SDS-PAGE and visualized by autoradiography (Fig. 4b). As previously shown [10] GST-Cdc25 was phosphorylated by 6his-Chk1. GST-Cdc25 was also phosphorylated by 6his-Cds1 *in vitro*. Experiments were performed to determine if Cdc25 phosphatase activity was effected by phosphorylation. In one set of experiments GST-Cdc25 was assayed by its ability to activate the H1 kinase activity of Cdc2/Cyclin B immune-precipitates. Phosphorylation of GST-Cdc25 by 6his-Cds1 or by 6his-Chk1 inhibited the ability of GST-Cdc25 to activate Cdc2/Cyclin B (Fig. 4d). Inhibition was dependent on the presence of ATP, was seen at a molar ratio of 1/100 kinase to GST-Cdc25, and was reversed by treatment of GST-Cdc25 with protein phosphatase 2A (PP2A) (Fig 4d). These results were somewhat surprising in light of previous reports that Chk1 does not inactivate Cdc25 [35] and that Cds1 phosphorylates Wee1 in fission yeast [36]. We therefore used second assay system, to monitor Cdc25 activity, in which dephosphorylation of Cdc2 was monitored by the disappearance of the slower migrating species of Cdc2 on gel-mobility analysis (Fig. 4e). In these assays Cdc25 activity was measured in the presence of 10mM EDTA and the absence of ATP, conditions that eliminate the possibility of 6his-Chk1 or 6his-Cds1

phosphorylating Cdc2 or Cyclin B. GST-Cdc25 catalyses a reduction in the slower migrating phosphorylated forms of Cdc2. Prior phosphorylation of GST-Cdc25 by 6his-Chk1 leads to a dose-dependent reduction in GST-Cdc25 activity (Fig. 4e). These data confirm genetic predictions that Chk1 negatively regulates Cdc25 activity [37, 38] and extend them by showing that the negative regulation involves inactivation of the phosphatase activity and that a second checkpoint kinase also phosphorylates and inactivates Cdc25. Chk1 has been shown to phosphorylate Cdc25 on serine-216 creating a binding site for 14-3-3 proteins [10, 35]. In this study inhibition of Cdc25 by Cds1 and Chk1 was seen without addition of 14-3-3 proteins, suggesting that 14-3-3 binding is not required for inhibition of Cdc25 by Chk1 or Cds1 *in vitro*. Furthermore similar experiments using a mutant form of GST-Cdc25 in which serine 216 has been replaced by the non-phosphorylatable alanine residue show that GST-Cdc25A216 is phosphorylated and inhibited by Cds1 and by Chk1 (data not shown). Experiments to determine what other sites on Cdc25 are phosphorylated by these kinases are in progress.

Cds1 is modified in response to DNA damage

Having determined that 6his-Cds1 inactivates Cdc25 *in vitro* and that Cdc25 is inactivated *in vivo* following DNA damage, we were interested in determining whether DNA damage might lead to modification or activation of human Cds1. Antiserum raised against 6his-Cds1 was used in immune-complex kinase assay using HeLa cells lysates, (Fig. 5). A weak signal corresponding to HsCds1 was detected in the sample from asynchronous HeLa cells, increased phosphorylation of HsCds1 was seen following irradiation. That this band represents Cds1 and not an associated protein was confirmed by re-precipitation of the protein following denaturation in 4% SDS (not shown). The *in vitro* phosphorylation of Cds1 most likely represents autophosphorylation, in that case the increased signal reflects an increase in activity following irradiation, however, the possibility that the signal is due an associated kinase cannot be excluded. Notwithstanding this, the increased *in vitro* phosphorylation of p64^{Cds1} suggests, that like Rad53 and Dun1 [39-41], HsCds1 is modified in response to DNA damage. Fission yeast Cds1 has recently been shown to be activated in response to incompletely replicated DNA [36, 42] we therefore examined the consequences of arresting DNA synthesis on the phosphorylation of p64^{Cds1}. HsCds1 from replication arrested cells behaved exactly like the protein from asynchronous cultures, no significant increase in phosphorylation was seen in response to thymidine or other agents that block DNA replication (Fig. 4 and data not shown). Increased phosphorylation of p64^{Cds1} was

detected following irradiation of thymidine arrested cells. Finally, the effect of damaging DNA in cells that are predominantly arrested outside S-phase was tested. Cells were cultured in the presence of nocodazole for 20 hour prior to irradiation. Again a weak, but detectable signal, was seen in the unirradiated sample, whilst irradiation of nocodazole arrested cells lead to increased phosphorylation. These findings contrast with the situation in fission yeast, in suggesting a role for human Cds1 in the DNA damage checkpoint rather than the replication checkpoint.

This study was based, in part, on the premise that checkpoint control proteins are conserved between organism. That assumption has facilitated the cloning of a number of human checkpoint genes [10 and this work]. However, despite structural homology, some these proteins may not be true functional homologs. For example, in fission yeast, *chk1* mutants are highly sensitive to DNA damage and are resistant to agents that block DNA replication [43] whereas mutants in the homologous *Drosophila* gene, *grapes*, respond normally to DNA damage but are sensitive to delayed DNA synthesis. [44, 45]. Likewise for Cds1, fission yeast mutants are sensitive to agents that block replication but have normal responses to DNA damage [12], whereas the budding yeast homologs, Rad53 and Dun1, have functions in both the replication and DNA damage responses [39, 46]. In this study we have defined a mechanism by which both human Cds1 and Chk1 might contribute to the G2/M checkpoint, the possibility that Cds1 carries out some of the other checkpoint/repair functions undertaken by its structural homologs remains to be explored.

7) Conclusion

More than 50% of breast cancers are defective in p53 function. These cells lack the G1 checkpoint that normally arrests cells in the presence of damaged DNA, they are therefore largely dependent on the G2 checkpoint for survival. By understanding the mechanisms by which the G2 checkpoint operates in human cells we expect to provide the information needed to manipulate the function of this checkpoint in therapy. The results obtained from our analysis of the over-expression of non-phosphorylatable Cdc2 in HeLa cells suggested that the regulation of the inhibitory phosphorylation of Cdc2 was likely to be an important component of the G2 checkpoint in human cells. We therefore continued with experiments aimed at showing whether Wee1/Myt1 or Cdc25 activity is altered in response to DNA damage. We found that Cdc25 activity is reduced following irradiation and have identified two human kinases which can phosphorylate and inactivate Cdc25 in vitro. Both of these kinases are modified in response to DNA damage. The function of these enzymes is likely to be critical in determining viability when cells are exposed to agents that damage DNA. Cds1 and Chk1 therefore represent attractive target enzymes in the search for improved anti-cancer therapy.

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APPENDICES

Methods

Cell lines

Normal (GM637G) and AT-deficient (GM5849C) SV40 transformed human fibroblasts obtained from Coriell Institute for Medical Research, Camden NJ. were grown in D-MEM supplemented with 15% fetal bovine serum, 100µg/ml penicillin and streptomycin. For γ -irradiation experiments cells were irradiated with a cesium¹³⁷ source in a Gamma Cell 1000 at a rate of 3.8 Gy/minute. For UV-irradiation experiments cells were washed once with PBS and irradiated at 254 nm in a UV Stratalinker 1800.

Assays and Western Analysis

Cells were lysed in ice-cold lysis buffer (50mM Tris pH 7.4 containing 2mM magnesium chloride, 1 mM phenylmethanesulphonyl fluoride, and 5 µg/ml leupeptin, pepstatin and aprotinin). Lysates were cleared by centrifugation at 10,000 x g for 10 minutes and the protein concentration of the supernatants determined using the Lowry assay. Dephosphorylation of Cdc2 was initiated by addition of 10mM EDTA and incubation at 30°C. At the indicated time the activity of Cdc2/Cyclin B was assayed by measuring the histone H1 kinase activity present in anti-Cyclin B immune-precipitates [4]. For immunoblots 400µg of cell lysate was immune-precipitated using anti-Cyclin B antibody, resolved on an 11% acrylamide-SDS gels. Monoclonal antibody against the PSTAIRE motif of Cdc2 was used to detect the different phospho-forms of Cdc2.

Recombinant baculo-viruses and protein production

Recombinant viruses encoding 6his-Chk1, 6his-Cds1, 6his-Wee1, 6his-Myt1, 6his-Cdc2 and GST-Cdc25C were generated using the Bac-to-Bac expression system from Gibco/BRL. 6his-fusion proteins were purified following the procedure described in [47]. GSH sepharose beads were incubated for 15 minutes in Sf9 extracts the beads were collected by centrifugation and washed three times with lysis buffer (50mM Tris pH 8.0, 5mM EDTA, 150mM NaCl, 0.1% NP40, 5% glycerol, 0.1% β -mercaptoethanol and protease inhibitors). Beads were washed two-times with kinase assay buffer (50mM Tris pH7.4 10mM MgCl₂) prior to phosphorylation reactions or two times with phosphatase assay buffer (50mM Imidazole pH 7.4, 5mM EDTA and 0.1% β -mercaptoethanol) prior to phosphatase assays. Phosphorylated Cdc2 was

purified from Sf9 cells that had been simultaneously infected with recombinant baculoviruses encoding 6his-Cdc2, 6his-Wee1, 6his-Myt1 and GST-Cyclin B [5]. 6his-Cdc2 complexed to Cyclin B was purified using GSH beads using the conditions described for GST-Cdc25 except that 1mM VO_4 was included in the lysis buffer. The catalytic subunit of Protein phosphatase 2A was purified from rabbit skeletal muscle [48]. Western analysis showed that quadruple infection resulted in phosphorylation of the majority of Cdc2/GST-CyclinB at one or both inhibitory sites.

Cloning

A search for sequences similar to *S. pombe cds1+* was carried out using the TBLASTN program. A human expressed sequence tag (EST) cDNA clone (No. 864164) was identified in the proprietary LifeSeq® database (Incyte Pharmaceuticals Inc., Palo Alto, USA). Sequence analysis of the 1.3 kb insert revealed an incomplete open reading frame which was highly similar to the *S. pombe cds1*. Approximately 650 nucleotides of novel 5' DNA sequence were obtained by 5' RACE. Termination codons were present in all three reading frames in the 120 nucleotides immediately 5' to the putative HsCds1 initiation codon, indicating that the complete coding region had been isolated. The sequence shown here is identical to two partial sequences in the National Centre for Biotechnology Information (NCBI) databases. The EST (AA285249) and genomic sequence (H55451) most likely, encode the same protein product.

A human expressed sequence tag encoding a protein with homology to *S. pombe chk1* [33] and to *Drosophila grapes* [45] was identified by BLAST analysis of the NCBI maintained database. Clones were retrieved from ATCC (IMAGE Clone ID 663485) and sequenced. The human Chk1 homologue used here is identical to the one described by Sanchez et al. [10], with the exception of three amino-acid substitutions (H/N at amino acid 64, R/H at 141 and N/M at 410).

Antibodies

Antibodies to HsCds1 were generated by immunizing a rabbit with 6his-Cds1 purified from Sf9 cells according to [49]. The resulting sera immune-precipitates and active kinase of the expected molecular weight from Sf9 cells infected with 6his-Cds1 virus but not from uninfected Sf9 cells or from cells infected with 6his-Chk1 virus (not shown).

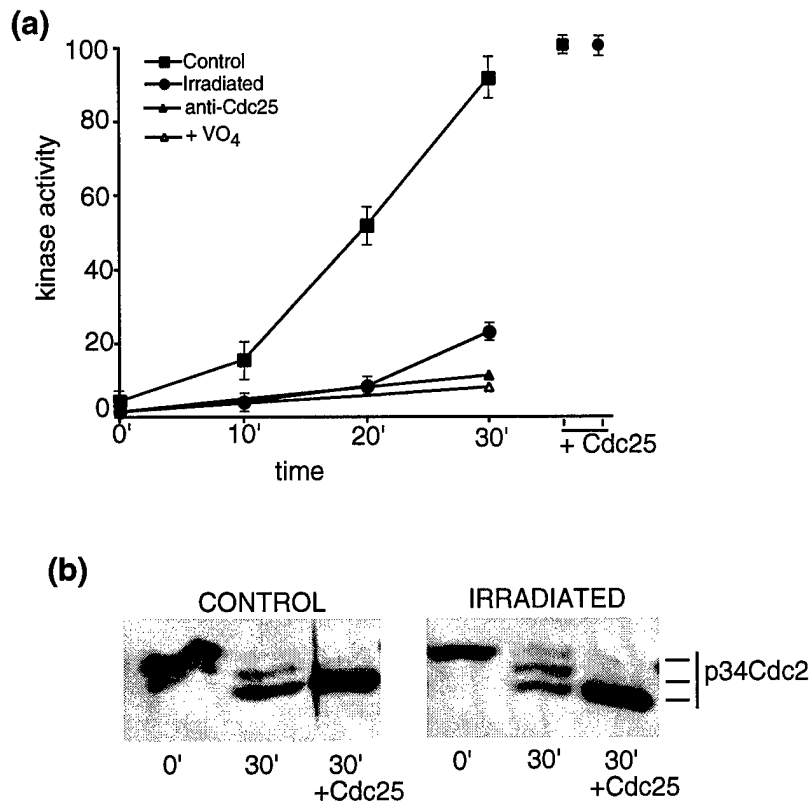


Figure 1. Cdc25 is down-regulated in response to DNA damage. (a) Cultures of asynchronous HeLa cells were divided and either mock treated or irradiated with 10 Gy 1 hour prior to harvesting. Cell lysates were prepared and the dephosphorylation reaction was initiated by addition of 10mM EDTA. At the indicated times Cdc2/Cyclin B was immune-precipitated and histone H1 kinase activity was assayed [4]. Complete dephosphorylation and activation was obtained by addition of GST-Cdc25c purified from baculo-virus infected insect cells. H1 kinase activity was normalized to the plus GST-Cdc25 control. Results are the average of three data sets and are representative of 10 separate experiments. (b) The phosphorylation state of Cdc2 was determined by immune-blotting of Cyclin B immune-precipitates.

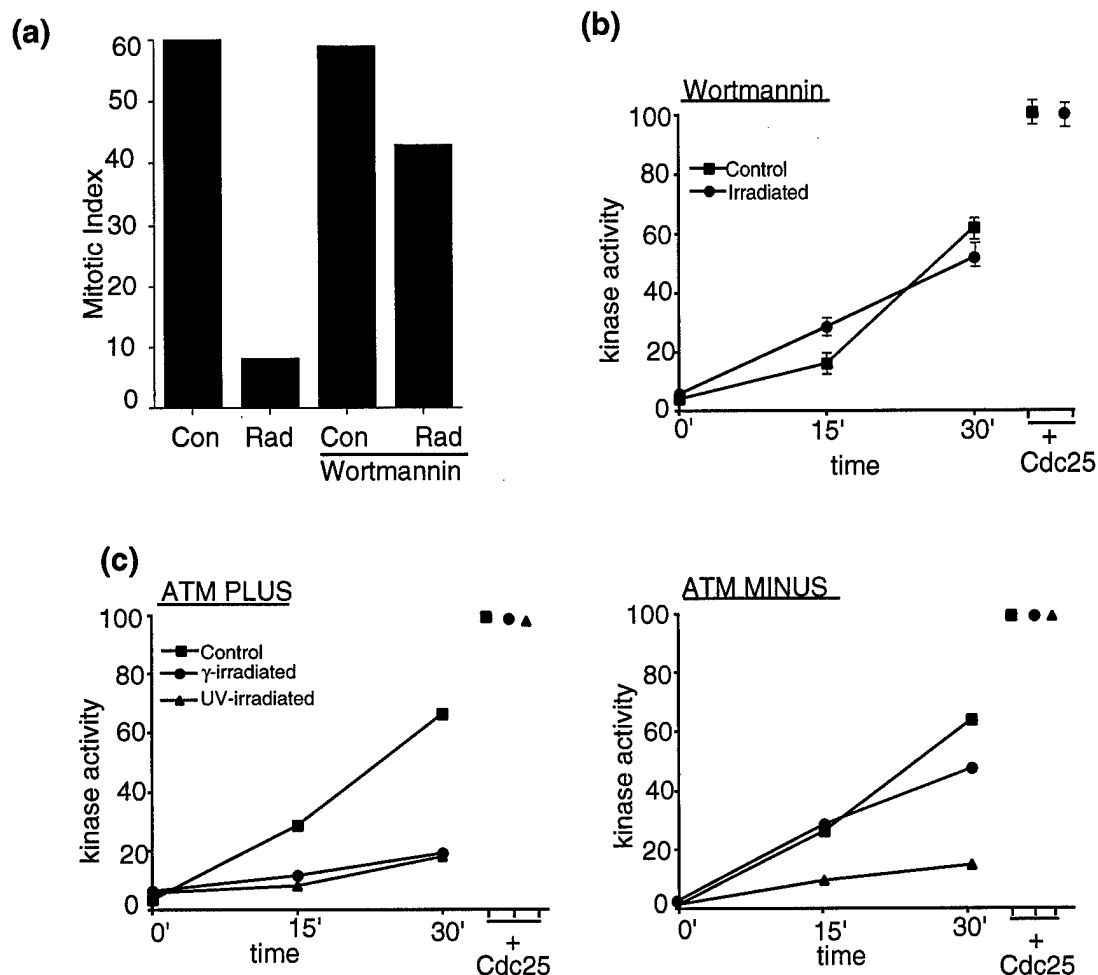


Figure 2 . Radiation induced inactivation of Cdc25 requires the activity of PI 3-like kinases.

(a) Wortmannin over-rides the radiation induced delay in entry to M-phase. Cells were cultured in 10 μ M wortmannin for 1 hour prior to either mock treatment or irradiation with 10 Gy. 100ng/ml nocodazole was added and 14 hours later cells were fixed in 70% ethanol, stained with Hoechst and mitotic index was scored. (b) Wortmannin prevents the irradiation induced down-regulation of Cdc25. Cells were cultured in the presence of 10 μ M wortmannin for 1 hour prior to either mock treatment or irradiation, extracts were prepared and assayed as in Figure 1. (c) The ATM gene is required for the down-regulation of Cdc25 activity following γ -irradiation but not UV-irradiation. The data is the average of two data sets and is representative of 3 independent experiments.

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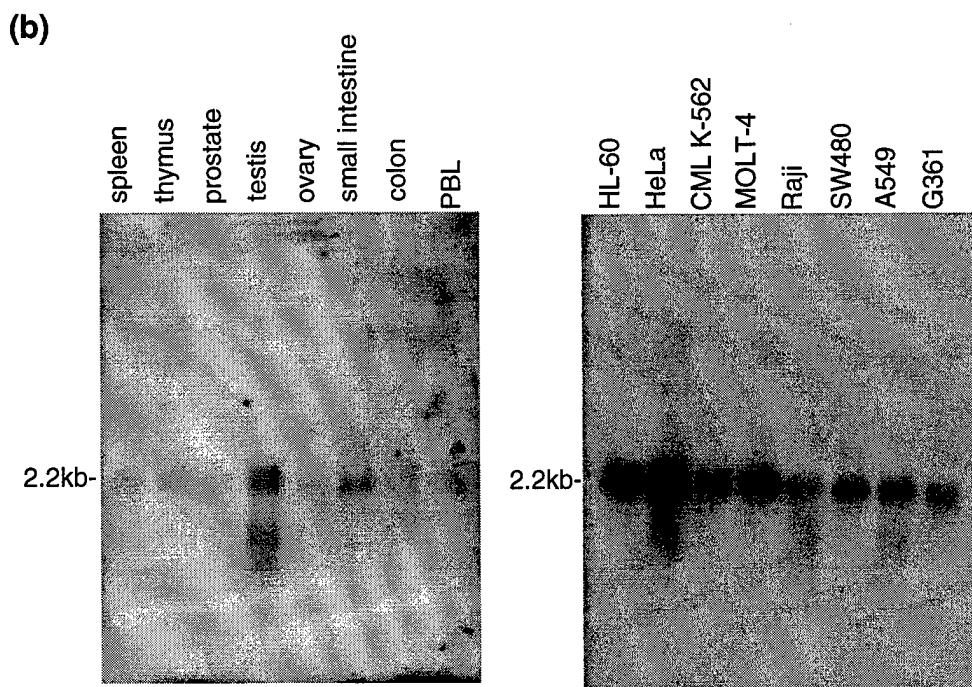


Figure 3 Identification of a human homologue of Cds1

(a) A human cDNA encoding an open reading frame of 534 amino acids with similarity to checkpoint kinases from *S. pombe* and *S. cerevisiae* was identified. Alignment of Cds1 homologs was generated using CLUSTALW (page 21). Amino acid identities are boxed, conservative changes are shaded. Putative Fork-head associated domains are under-lined. Human Cds1 is HsCds1, Sp cds1 is a checkpoint kinase from the fission yeast *S. Pombe*, RAD53 and DUN1 are checkpoint kinases from the budding yeast *S. Cerevisiae*. (b) Northern analysis of human Cds1. Blots containing 2µg polyadenylated RNA from the indicated tissues or cell-lines were probed with HsCds1.

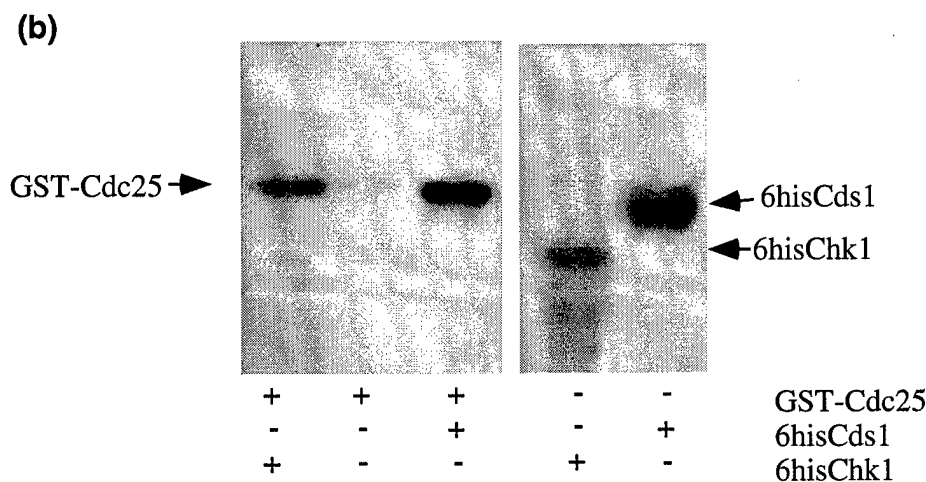
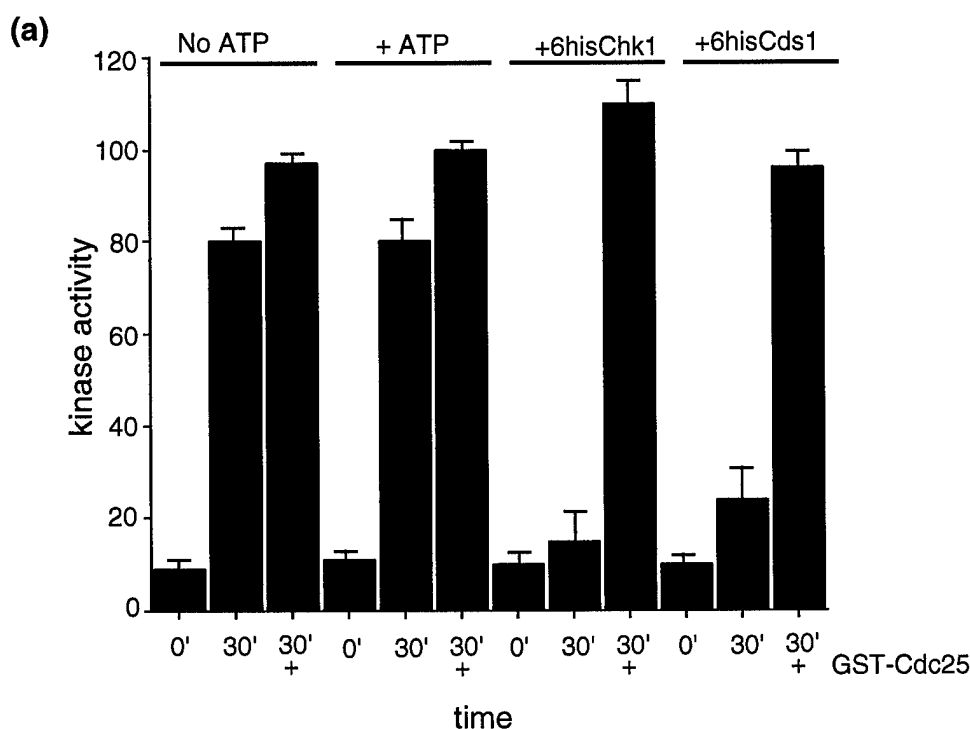


Figure 4 6his-Cds1 and 6his-Chk1 phosphorylate and inhibit Cdc25 *in vitro*.

(a) 6his-Cds1 and 6his-Chk1 down regulate dephosphorylation of Cdc2 in HeLa cell extracts. Recombinant 6his-Cds1 or 6his-Chk1 purified from insect cells was incubated with HeLa cells extracts in the presence of an ATP-regenerating system for 30 min. at 30°C [50]. Control extracts incubated at 30°C in the presence of ATP (+ ATP) or maintained on ice for 30 min. (No ATP) show similar kinetics of activation. Dephosphorylation and activation of Cdc2/cyclin B was initiated by addition of excess EDTA at the zero time point. Numbers are the average of three experiments. (b) GST-Cdc25 purified from insect cells was incubated with either 6his-Cds1, mock beads or 6his-Chk1 in the presence of [γ - 32 P] ATP for 15 min. at 30°C. GST-Cdc25 bound to GSH-sepharose was washed 3 times prior to addition of SDS. Proteins were resolved by SDS-PAGE and visualized by autoradiography. The authenticity of each band was confirmed by re-precipitation of the labelled bands with the relevant sera.

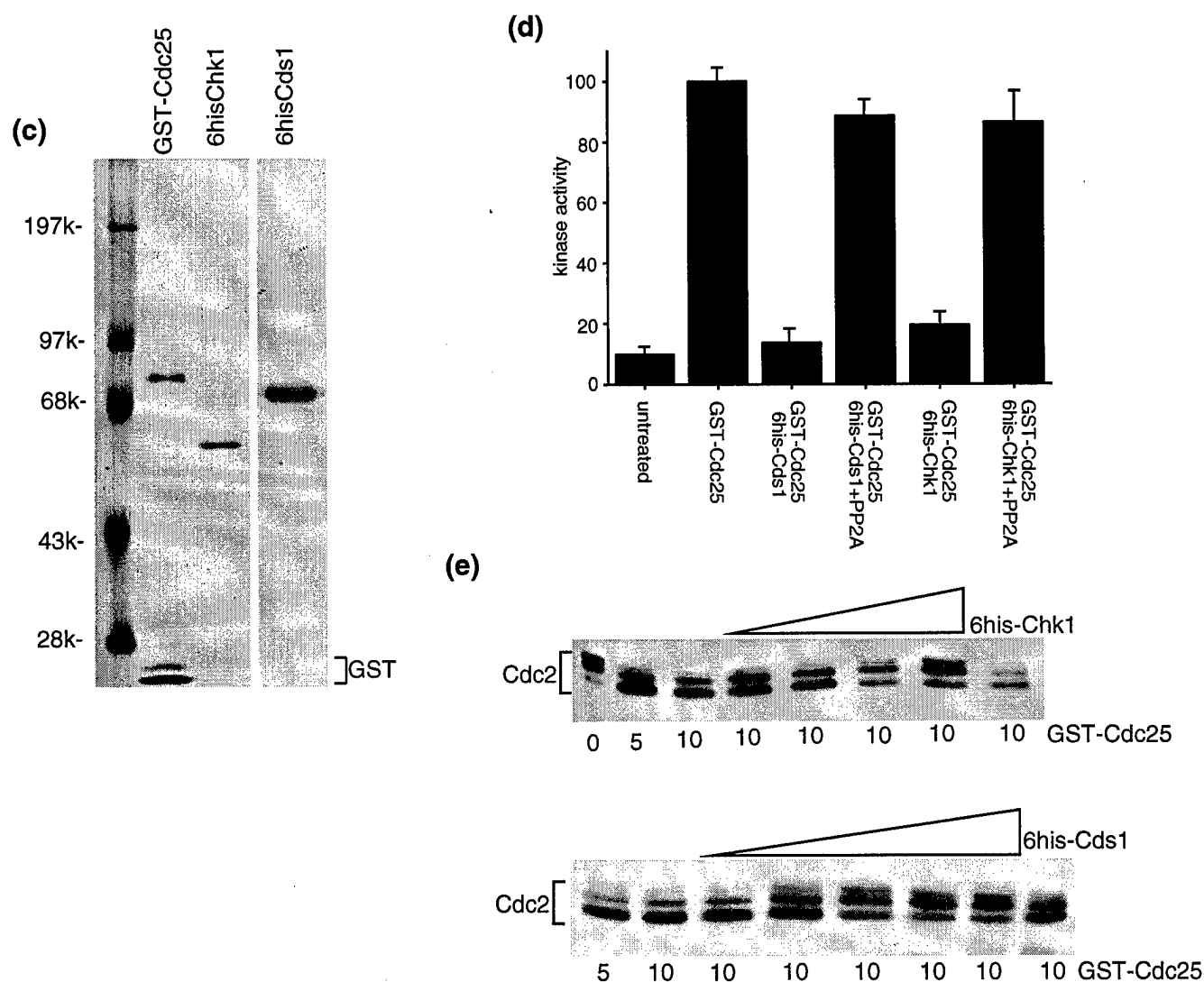


Figure 4 6his-Cds1 and 6his-Chk1 phosphorylate and inhibit Cdc25 in vitro.

(c), Affinity purified GST-Cdc25, 6his-Chk1 and 6his Cds1 were resolved by SDS-PAGE and visualized by Coomassie blue staining. (d), 6his-Cds1 or 6his-Chk1 inhibit purified GST-Cdc25. GST-Cdc25c from insect cells was incubated with 6his-Cds1, 6his-Chk1 or buffer in the presence of 1mM ATP for 30 min. at 30°C. The sample was split and either incubated alone or with PP2A for 30 minutes at 30°C. GST-Cdc25 was washed three times with phosphatase buffer containing 0.4μM microcystin prior to incubation with Cdc2/Cyclin B immune-precipitates from asynchronous HeLa cells. The H1 kinase activity of Cdc2/Cyclin B was determined. (e), Reduced GST-Cdc25 activity after phosphorylation by 6his-Chk1 or by 6his-Cds1. GST-Cdc25 was assayed by its ability to convert Cdc2 to the faster migrating-dephosphorylated form. In the presence of GST-Cdc25 the majority of Cdc2 is converted to the lower band of the triplet. Prior phosphorylation of GST-Cdc25 by 6his-Chk1 or 6his-Cds1 reduced the appearance of the dephosphorylated form. GST-Cdc25 was phosphorylated with increasing quantities of each kinase. Numbers refer to relative quantities of proteins added and are arbitrary. Results are representative of 5 independent experiments.

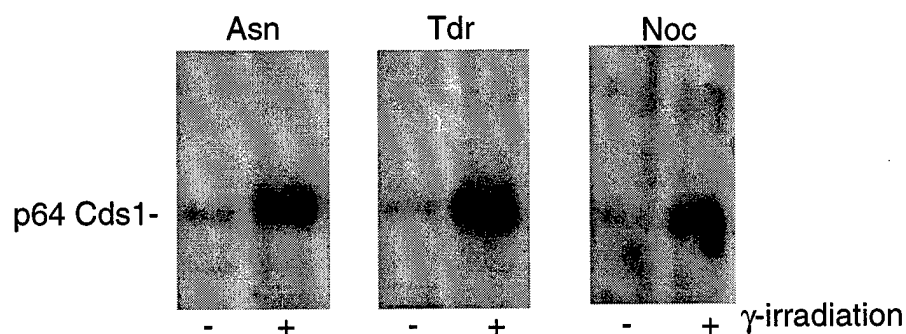


Figure 5. Modification of HsCds1 in response to DNA damage.

Anti-Cds1 immune-precipitates prepared from 400 μ g of HeLa cell extract were incubated in the presence of 10 μ Ci[γ -32P] ATP for 15 min. at 30°C. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Cells were accumulated with unreplicated DNA by addition of 2mM thymidine for 17 hours, Tdr. Cells were accumulated in M-phase by growth in the presence of 100ng/ml nocodazole for 18 hours. Where indicated cultures were exposed to 10 Gy of ionizing radiation 1 hour prior to harvesting.

Publications

Blasina, A., Paegle, E.S., and McGowan, C.H., **The Role of Inhibitory Phosphorylation of CDC2 following DNA Replication Block and Radiation Induced Damage in HeLa Cells.** *Mol. Biol. Cell*, 1997. 8: 1-11.

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Blasina, A., Van de Weyer I., Laus, MC., Luyten, W.H.M.L.,

Parker, A.E., and McGowan, C.H. **A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25.** *Current Biology* submitted.

Personnel.

The following personnel received pay from this effort.

C.H. McGowan

M. Teasdale

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B.W. Lee